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The Yeast Hex3·Slx8 Heterodimer Is a Ubiquitin Ligase Stimulated by Substrate Sumoylation*[S]

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Hex3 and Slx8 are *Saccharomyces cerevisiae* proteins with important functions in DNA damage control and maintenance of genomic stability. Both proteins have RING domains at their C termini. Such domains are common in ubiquitin and ubiquitin-like protein ligases (E3s), but little was known about the molecular functions of either protein. In this study we identified *HEX3* as a high-copy suppressor of a temperature-sensitive small ubiquitin-related modifier (SUMO) protease mutant, *ulp1ts*, suggesting that it may affect cellular SUMO dynamics. Remarkably, even a complete deletion of *ULP1* is strongly suppressed. Hex3 forms a heterodimer with Slx8. We found that the Hex3·Slx8 complex has a robust substrate-specific E3 ubiquitin ligase activity. In this E3 complex, Slx8 appears to bear the core ligase function, with Hex3 strongly enhancing its activity. Notably, SUMO attachment to a substrate stimulates its Hex3·Slx8-dependent ubiquitination, primarily through direct noncovalent interactions between SUMO and Hex3. Our data reveal a novel mechanism of substrate targeting in which sumoylation of a protein can help trigger its subsequent ubiquitination by recruiting a SUMO-binding ubiquitin ligase.

Ubiquitin and small ubiquitin-related modifier (SUMO)[5] are highly conserved proteins that can be covalently attached to target proteins (1–3). Both ubiquitin and SUMO are attached to substrates by three types of enzymes: E1, E2, and E3. These enzymes work consecutively to catalyze isopeptide bond formation between the C terminus of ubiquitin or SUMO and a lysine side chain of the acceptor protein. In the case of ubiquitination, ubiquitin is first activated by the E1 activating enzyme and is transferred from a thiol group on E1 to a thiol on an E2 conjugating enzyme. The final step of ubiquitin conjugation to target proteins is facilitated by an E3 ubiquitin ligase, which binds both E2 and substrate and facilitates ubiquitin transfer to the substrate. E3s are key mediators of substrate specificity.

Sumoylation happens in an analogous manner to ubiquitin conjugation, requiring an E1, E2, and usually an E3, of which only a handful have been identified so far (4, 5). Unlike ubiquitination, which often targets substrates for proteasomal degradation, SUMO modification is not believed to target substrates directly to the proteasome. Instead, sumoylation appears to modulate the functional properties of a protein, including its localization, activity, and interactions with other macromolecules.

Similar to ubiquitin regulation, SUMO-protein conjugates are in a dynamic state, with specialized proteases rapidly removing the SUMO moieties. In *Saccharomyces cerevisiae*, Ulp1 and Ulp2 are the only known SUMO proteases and are responsible for desumoylating distinct sets of SUMO-modified proteins (6, 7). Ulp1 is also required for proteolytic maturation of the SUMO precursor encoded by the *SMT3* gene. Like SUMO itself and the SUMO E1 (the Aos1-Uba2 heterodimer) and E2 (Ubc9), Ulp1 is essential for yeast cell viability. The temperature-sensitive *ulp1ts* mutant grown at nonpermissive temperature arrests primarily in the G_2/M phase of the cell cycle (7).

To understand the mechanistic basis of *ulp1ts* lethality and gain insight into Ulp1 regulation of SUMO dynamics, we screened a high-copy yeast genomic library for dosage suppressors of *ulp1ts*. From this screen we isolated the *HEX3* gene. *HEX3* (*SLX5*) was originally identified together with *SLX8* as a gene that becomes essential for viability in the absence of the Sgs1/Top3 pathway (8). Hex3 and Slx8 form a heterodimer and localize to the nucleus (9). Deletions of either or both genes display similar defects in genome stability and are sensitive to DNA-damaging agents, suggesting that they work interdependently to suppress the accumulation of DNA damage (10). Hex3 and Slx8 each bear a zinc-coordinating RING domain, a motif characteristic of many ubiquitin and SUMO ligases (E3s). Hex3 also binds SUMO *in vivo* (11), suggesting that the Hex3·Slx8 complex might function by regulating SUMO dynamics. This hypothesis was further supported by the discovery that mutations in *HEX3* and *SLX8*, like mutations in many known components of the SUMO pathway, are able to suppress a temperature-sensitive mutation of the essential Mot1 transcriptional regulator (12).

Here we report that the Hex3·Slx8 heterodimer is a ubiquitin ligase. Recombinant Slx8, but not Hex3, exhibits RING-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[5] The abbreviations used are: SUMO, small ubiquitin-related modifier; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein ligase; SIM, SUMO-interacting motif; ORF, open reading frame; WT, wild type; MBP, maltose-binding protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TEV, tobacco etch virus.

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dependent auto-ubiquitinating activity. We identified Rad52, a protein that regulates homologous recombination-dependent DNA repair, as a model *in vitro* substrate for Hex3·Slx8. Slx8 by itself has a very weak Rad52-ubiquitin ligation activity that is strongly stimulated by Hex3. Both the Slx8 and Hex3 RING domains are required for this activity and for *in vivo* DNA damage control. Notably, SUMO attachment to Rad52 stimulates its Hex3·Slx8-dependent ubiquitination and enhances binding between Hex3 and the substrate. Hex3 binds SUMO noncovalently, primarily through two N-terminal SUMO-interacting motifs (SIMs).

Our data demonstrate that Hex3 and Slx8 work together as a ubiquitin E3 in which Slx8 appears to provide the core ligase function and Hex3 enhances Slx8 activity and substrate targeting, in part by binding SUMO in SUMO-protein conjugates. This novel mechanism of ubiquitin-substrate specificity may also be relevant to the human BRCA1-BARD1 ubiquitin ligase implicated in human breast cancer, which also is a heterodimer of two RING proteins with asymmetric mechanistic contributions from the two RING domains (13, 14).

EXPERIMENTAL PROCEDURES

Yeast and Bacterial Methods—Yeast rich (yeast extract/peptone/dextrose) and minimal (SD) media were prepared as described previously, and standard methods were used for genetic manipulation of yeast (15). Standard techniques were used for recombinant DNA work in *Escherichia coli*.

Yeast Strains—The *hex3Δ::kanMX4* and *slx8Δ::kanMX4* strains were obtained from Open Biosystems (Huntsville, AL). They are in the BY4741 strain background. Where indicated, *kanMX4* was PCR-amplified with primer pairs corresponding to regions 200 nucleotides upstream and downstream of the *HEX3* and *SLX8* open reading frame (ORF), respectively. The deletion cassettes were then transformed into wild-type (WT) strain MHY501 (16) to generate the *hex3Δ::kanMX4* (MHY3712) and *slx8::kanMX4* (MHY3716) strains. The *hex3Δ::kanMX4 slx8Δ::kanMX4* double deletion strain (MHY3861) was generated from a cross between the two single mutants.

Plasmids and Mutagenesis—Genomic *SLX8*, containing the endogenous promoter and terminator, was PCR-amplified and cloned into different plasmids. Genomic *HEX3* was obtained from the *ulp1ts* suppression screen described below, and various fragments were subcloned into the desired plasmids. YCplac22-ulp1ts-kanMX4 contains the *ulp1ts* allele present on a BamHI/PstI fragment. The *kanMX4* marker was inserted into the AflII site (after filling in the overhang with Klenow DNA polymerase) present 213 base pairs after the stop codon of *ulp1ts*. To generate FLAG-tagged Hex3 and T7-tagged Slx8, FLAG (DYKDDDDKG) and T7 (MASMTGGQQMG) epitope tags were introduced using PCR-mediated site-directed mutagenesis. To overexpress and purify Hex3 and Slx8 from bacteria, both ORFs were PCR-amplified and cloned into pMALc-HT (a gift from Sean Prigge, JHSOM), thereby adding an in-frame N-terminal maltose-binding protein (MBP) module followed by a TEV protease cleavage site and a His_6 epitope tag. For the corresponding RING-truncated versions, the PCR reactions were designed to introduce a stop codon at the position of the codon for the last zinc-coordinating Cys residue.

To generate a RING-truncated Hex3 protein for expression in yeast, a premature stop codon was introduced such that the last two conserved zinc-coordinating cysteines were deleted. Specifically, the plasmid pRS425-HEX3 was digested with the restriction enzyme BspE1, filled in with Klenow polymerase, and religated to create an allele encoding a truncated Hex3 protein ending at Pro-591 followed by Ala-Gly and a stop codon. For creating point mutations in the RING domain, Cys-561 and Cys-564 of Hex3 and Cys-206 and Cys-209 of Slx8 were changed to serines by QuikChange mutagenesis (Stratagene). Rad52 clones for *E. coli* overexpression were made by inserting the PCR-amplified *RAD52* ORF into pET11d and pET21a (for T7 epitope tagging; Novagen). A RAD52-SUMO *E. coli* overexpression clone was generated by inserting the PCR-amplified *SMT3* ORF (lacking the region encoding the C-terminal Gly-Gly) in-frame with and downstream of *RAD52* in pET21a-T7-RAD52. In this fusion construct, the major *in vitro* Rad52 sumoylation site (Lys-220)[6] was changed to arginine by QuikChange mutagenesis. For pQE30-TEV-SMT3gg, DNA encoding the mature portion of SUMO (Smt3) was amplified by PCR and cloned into pQE30 (Qiagen) along with a primer-encoded N-terminal TEV cleavage site.

Isolation of HEX3 by ulp1ts Suppression—A *ulp1ts* strain was generated by replacing the WT *ULP1* plasmid in MHY1321, which lacks the chromosomal copy of *ULP1* (17), with YCplac22-ulp1ts-kanMX4. The strain was co-transformed with a plasmid expressing mature SUMO (pRS426-GPD-FLAG-SMT3gg) (11). The resulting strain was transformed with a yeast genomic DNA library carried in the 2μ-*LEU2* vector YEp13 (18). Approximately 10,000 Leu+ transformants were isolated at 30 °C and tested for their ability to grow at the nonpermissive temperature of 37 °C upon replica plating. Of the 8 clones that supported growth at 37 °C, 6 carried 5′ truncated or full-length *ULP1* genes. The other two plasmids contained identical DNA inserts from chromosome XVI that included the *HEX3* ORF. A SacI/PacI restriction fragment from the original isolate containing only the *HEX3* ORF was cloned into the high-copy plasmids pRS425 and pRS423. This fragment was found to be sufficient for full *ulp1ts* suppression.

Yeast Two-hybrid Assays—Yeast two-hybrid strains containing pOAD- and pOBD2-based plasmids with *HEX3* and *SMT3* were a gift from Marissa Vignali (Yeast Resource Center, University of Washington). We initially transformed the yeast two-hybrid reporter strain PJ69–4α that contained the pOBD2-SMT3 bait plasmid with pOAD-HEX3. Subsequently, pOAD-HEX3 was subject to PCR-mediated site-directed mutagenesis to generate the series of SIM point mutants (see "Results"). The resulting pOAD-HEX3-SIM mutants (*sim*-A, *sim*-B, *sim*-C, *sim*-D, and *sim*-A/B) were transformed into the yeast bait strain, and SUMO (Smt3) interaction was determined on SD plates lacking adenine. All putative SIM mutations were confirmed by DNA sequencing.

Recombinant Protein Purification—Hex3, Slx8, and their derivatives were expressed as MBP fusions in BL21 Star™

[6] L. Krejci and P. Sung, unpublished data.

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(DE3) cells containing plasmid pRIL, which expresses several rare-codon tRNAs (a gift from Sean Prigge, JHSOM, MD). Proteins were affinity purified on an amylose resin (New England Biolabs). Rad52 was expressed and purified according to Song and Sung (19). T7-tagged Rad52 and the T7-Rad52-SUMO fusion were expressed as above and purified on a Talon Superflow column (BD Biosciences). Bacterially expressed His_6-Ubc4 was purified as in Bays *et al.* (20), and yeast SUMO was purified from bacteria carrying pQE30-TEV-SMT3gg according to Johnson and Gupta (21).

Immunoprecipitation and Pulldown Assays—For SUMO pulldown experiments, 20 nM recombinant Hex3, Slx8, or MBP was incubated with SUMO-1-agarose (BostonBiochem) or, as a control, protein A-agarose (Repligen) in 0.5 ml of buffer containing 0.5% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture (Roche Applied Science) and 50 mM HEPES, pH 7.5. Proteins pulled down with beads were washed in the same buffer except with 1% Triton X-100 and 200 mM NaCl. For Rad52/Rad52-SUMO co-immunoprecipitation with Hex3, 10 nM recombinant Hex3, Slx8, or MBP were incubated with 10 nM T7-tagged Rad52 or Rad52-SUMO in 1 ml of buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture, 0.1 μM bovine serum albumin, and 50 mM HEPES, pH 7.5. After incubation with anti-T7 monoclonal antibody (Novagen), samples were precipitated by protein A-agarose. Precipitates were washed in the same buffer but containing 1 M NaCl.

In Vitro Auto-ubiquitination Assay—Based on the procedures used in Swanson *et al.* (22), a typical 20-μl reaction for Slx8 and Hex3 auto-ubiquitination contained 0.4 μg of yeast Uba1 (BostonBiochem), 0.2 μg of human His_6-Ubc4 (20), 2.5 μg of human recombinant ubiquitin (Boston Biochem), 10 mM ATP, and 2 μM recombinant wild-type Hex3, Slx8, or their respective RING mutants in reaction buffer (2.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 50 mM Tris·HCl, pH 7.5). Reactions were allowed to proceed at 30 °C for 2 h and were terminated by adding 3× SDS gel-loading buffer. The resulting ubiquitin conjugates were analyzed by immunoblotting with an anti-ubiquitin monoclonal antibody (Covance). To detect the ubiquitinated His_6-tagged proteins from the auto-ubiquitination assay, samples from the reaction were resolved on 8% SDS-PAGE gels and probed with an anti-His4 antibody (Qiagen).

In Vitro Rad52 Ubiquitination Assay—Unless otherwise noted, Rad52 and Rad52-SUMO ubiquitination assays contained 0.3 μg of yeast Uba1, 0.2 μg of human His_6-Ubc4, 1.2 μg of human recombinant ubiquitin, and 10 mM ATP in a 20-μl reaction as above. Different concentrations of recombinant Hex3, Slx8, and the respective RING mutants and of recombinant Rad52 or Rad52-SUMO were used between reactions and are reported in the figure legends. Ubiquitinated Rad52 and Rad52-SUMO were resolved on 8% SDS-PAGE gels and analyzed by immunoblotting with anti-Rad52 (23) or anti-T7 (Novagen) antibodies. For SUMO competition assays, recombinant Hex3 and Slx8 were preincubated with 10 μg of recombinant yeast SUMO in reaction buffer (100 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM dithiothreitol, 10% glycerol, and 50 mM Tris·HCl, pH 7.5) for 30 min at 4 °C. Reactions were transferred to 30 °C and initiated by adding ubiquitinating enzymes and substrate. Reactions were terminated at the designated times by the addition of 3× SDS gel-loading buffer.

In Vitro Sumoylation Assays—Following Johnson and Gupta (21), a 50-μl reaction for the auto-sumoylation assay contained the following bacterially expressed recombinant yeast proteins: 2 μg of SUMO, 1.1 μg of Aos1/Uba2 dimer, and 0.5 μg of Ubc9. Concentrations of recombinant Hex3 and Slx8 were 2.5 and 5 μM, respectively. Reactions were performed with 5 mM ATP, 50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 20 μg of bovine serum albumin at 30 °C and were terminated by boiling in SDS-gel loading buffer. Samples were resolved by 6–12% gradient SDS-PAGE and analyzed by anti-SUMO immunoblotting (6).

RESULTS

HEX3 Is a High-copy Suppressor of the ulp1ts and ulp1Δ Mutants—*ULP1* encodes an essential yeast desumoylating enzyme (7). The Ulp1 protease has both an isopeptidase activity, which cleaves SUMO from substrate lysines, and a SUMO-processing activity that removes the last three amino acids (ATY) from the SUMO precursor. A free di-glycine sequence at the mature SUMO C terminus is necessary for its conjugation to substrates. Deconjugation of SUMO from one or more unknown proteins by Ulp1 is required for progression through the G_2/M phase of the cell cycle (7).

We reasoned that sumoylation of such a critical substrate(s) could function to arrest cell cycle progression until certain other regulatory events had occurred, with subsequent activation or recruitment of Ulp1. Overexpression of the nonsumoylated substrate might, therefore, allow partial bypass of the *ulp1ts* block. High levels of other factors regulating G_2/M progression or Ulp1 or of another SUMO protease might also suppress the *ulp1ts* arrest. We, therefore, screened a high-copy yeast genomic library in *ulp1ts* cells to identify suppressors of the conditional growth defect of *ulp1ts* cells. From ~10,000 transformants, 8 clones capable of growth at 37 °C were isolated. Six carried plasmids with *ULP1* inserts. The remaining two suppressing plasmids had identical inserts, and the relevant gene was determined to be *HEX3* (*SLX5*).

HEX3 encodes a RING-domain protein involved in genome maintenance (8, 10). Importantly, the ability of *HEX3* to suppress the growth defect of *ulp1ts* at the nonpermissive temperature depended on co-expression of *SMT3gg*, encoding mature SUMO. Alone, neither *HEX3* nor *SMT3gg* was able to rescue the *ulp1ts* mutant, but the combination of both genes on separate high-copy plasmids supported robust growth of *ulp1ts* cells at 37 °C (Fig. 1*A*, *top panel*). This implied that high dosage *HEX3* specifically suppressed the SUMO isopeptidase defect of the *ulp1ts* mutant but did not restore SUMO-precursor processing. In support of this, provision of an *SMT3aty* expressing plasmid encoding the unprocessed SUMO precursor did not support growth of *ulp1ts* cells carrying high-copy *HEX3* (Fig. 1*B*). Remarkably, high-copy of *HEX3* was also capable of suppressing a complete deletion of *ULP1* both at 30 °C (Fig. 1*A*, *bottom panel*) and 37 °C (supplemental Fig. S1*A*). In contrast, increased Hex3 expression did not suppress the temperature-

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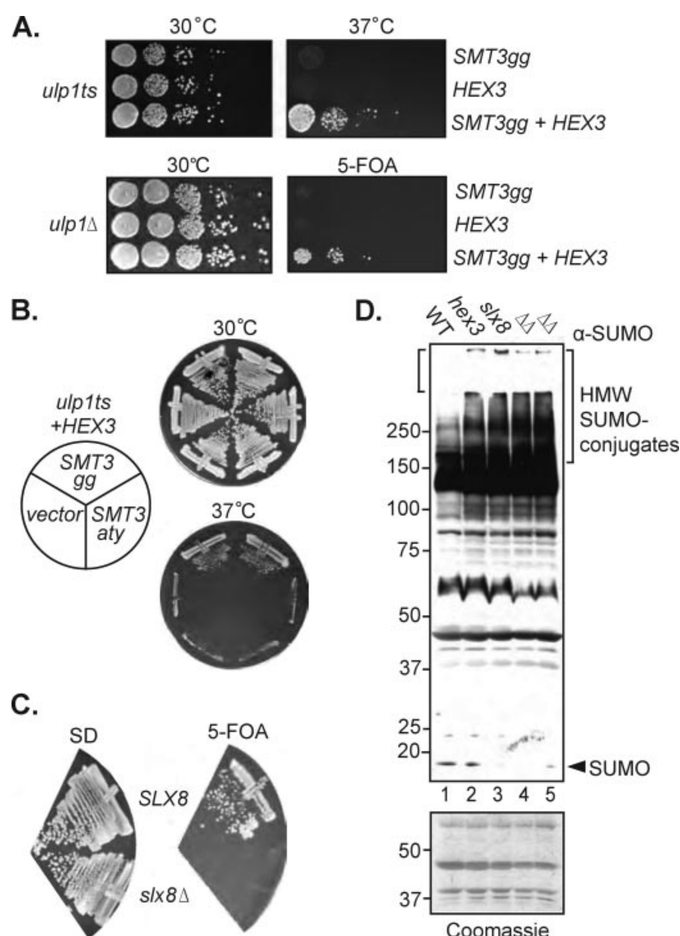

FIGURE 1. ***HEX3* is a high-copy suppressor of *ulp1* mutants and requires mature SUMO (Smt3gg) and *SLX8* for suppression.** *A*, *upper panels*, the *ulp1ts* strain was transformed with plasmids containing either *SMT3gg*, *HEX3*, or both, as indicated. After spotting cells in 10-fold serial dilutions, plates were incubated at 30 and 37 °C for 3 days. *Lower panels*, *A ulp1*Δ strain (MHY1321) expressing WT *ULP1* from a *URA3* plasmid was transformed with high-copy plasmids bearing the indicated genes. Transformants were spotted on minimal plates or plates containing 5-fluoro-orotic acid (*5-FOA*) and incubated at 30 °C for 3 days. 5-FOA selected against the *ULP1-URA3* plasmid. *B*, plasmids containing either no insert, *SMT3gg*, or *SMT3aty* (expressing the unprocessed precursor of SUMO) were transformed into *ulp1ts* cells carrying a high-copy *HEX3* plasmid. Transformants were streaked on plates and incubated at either 30 or 37 °C. *C*, *SLX8* was deleted from a yeast strain with a chromosomal deletion of *ULP1* but with a *ULP1-URA3* plasmid. Additional plasmids expressed mature SUMO and high levels of Hex3. *SLX8* and *slx8*Δ strains were streaked on minimal (SD) or 5-FOA plates and incubated at 30 °C for 3 days. *D*, SUMO conjugate levels in WT and mutant cells. Whole cell extracts from the indicated strains (ΔΔ, *hex3*Δ *slx8*Δ) were resolved by 5–12% gradient SDS-PAGE and analyzed by anti-SUMO immunoblotting. GelCode Blue staining of the SDS-PAGE gel showed comparable loading of proteins. Positions of free SUMO, high molecular weight (*HMW*) SUMO conjugates (*right bracket*), the stacking gel (*left bracket*), and molecular weight markers are indicated.

sensitive growth defect of *ulp2*Δ cells. *ULP2* encodes a second SUMO protease with distinct specificity and function (6).

SLX8 encodes another RING-containing protein that forms a heterodimer with Hex3 (8, 9). Both polypeptides are required for DNA damage resistance and maintenance of genome stability (8, 10). Although high-copy *SLX8* was unable to suppress the *ulp1ts* growth defect at 37 °C (data not shown), deletion of *SLX8* in a strain lacking *ULP1* prevented high-copy *HEX3* and *SMT3gg* from rescuing growth (Fig. 1*C*). These data are consistent with a role for both Hex3 and Slx8 in regulating SUMO-conjugate dynamics and suggest that Hex3, but not Slx8, is normally expressed at limiting levels.

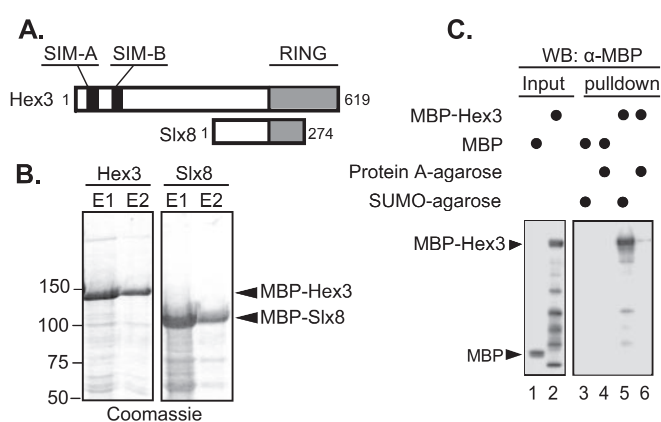

FIGURE 2. **Recombinant Hex3 interacts with SUMO *in vitro*.** *A*, schematic of Hex3 and Slx8 proteins with Hex3 SIMs and RING domains highlighted. *B*, purification of MBP fusions of Hex3 and Slx8 from *E. coli* cell extracts. Proteins were harvested from multiple elutions with maltose-containing buffer. Elutions 1 and 2 (*E1*, *E2*) were resolved by 8% SDS-PAGE. Size standards are indicated on the *left*. *C*, direct interaction between Hex3 and SUMO was determined by an *in vitro* pulldown assay. Recombinant MBP (New England Biolabs) and MBP-Hex3 were incubated with either SUMO-1-agarose or protein A-agarose. Precipitated proteins were run on 8% SDS-PAGE gels and detected by anti-MBP (Santa Cruz) immunoblotting (*WB*, *lanes 3–6*). *Lanes 1–2*, input (3.33%) MBP and MBP-Hex3.

Comparison of SUMO conjugates in WT, *hex3*Δ, *slx8*Δ, and *hex3*Δ *slx8*Δ double mutant (ΔΔ) cells by anti-SUMO immunoblotting revealed significantly greater levels of high molecular mass sumoylated species (greater than ~150 kDa, including material in the stacking gel) in the mutants relative to WT cells (Fig. 1*D*). Therefore, both Hex3 and Slx8 have a major role in limiting cellular SUMO-protein levels, consistent with previous observations (12). Because loss of these RING proteins causes an increase in SUMO conjugates, it is unlikely that they are SUMO ligases. They could function as components or activators of a SUMO protease or could reduce sumoylated protein levels by some other mechanism. We explore these possibilities below.

Recombinant Hex3 Binds to SUMO—Hex3 was previously reported to bind SUMO in yeast, and the interaction was insensitive to overexpressed Ulp1 protease (11), suggesting that Hex3 might associate with SUMO in a noncovalent manner. To test this, purified recombinant Hex3 fused with MBP (Fig. 2*B*, *left panel*) was incubated with human SUMO-1 coupled to agarose beads or, as a control, protein A-agarose. Proteins pulled down with the different resins were eluted and analyzed by anti-MBP immunoblotting (Fig. 2*C*). This assay revealed that MBP-Hex3, but not MBP alone, was able to bind specifically to SUMO. These data suggest that the effect of Hex3 on cellular SUMO dynamics may be based on a direct interaction between Hex3 and the SUMO polypeptide.

Interaction between Hex3 and SUMO Depends on SUMO-interacting Motifs in Hex3—From the Hex3 protein sequence, we identified a series of potential SIMs based on previously proposed SIM consensus sequences (11, 24, 25). A typical SIM features a patch of aliphatic residues often neighboring a cluster of acidic amino acids (26). Based on these criteria, we identified SIM-A and SIM-B in Hex3 together with two additional weak

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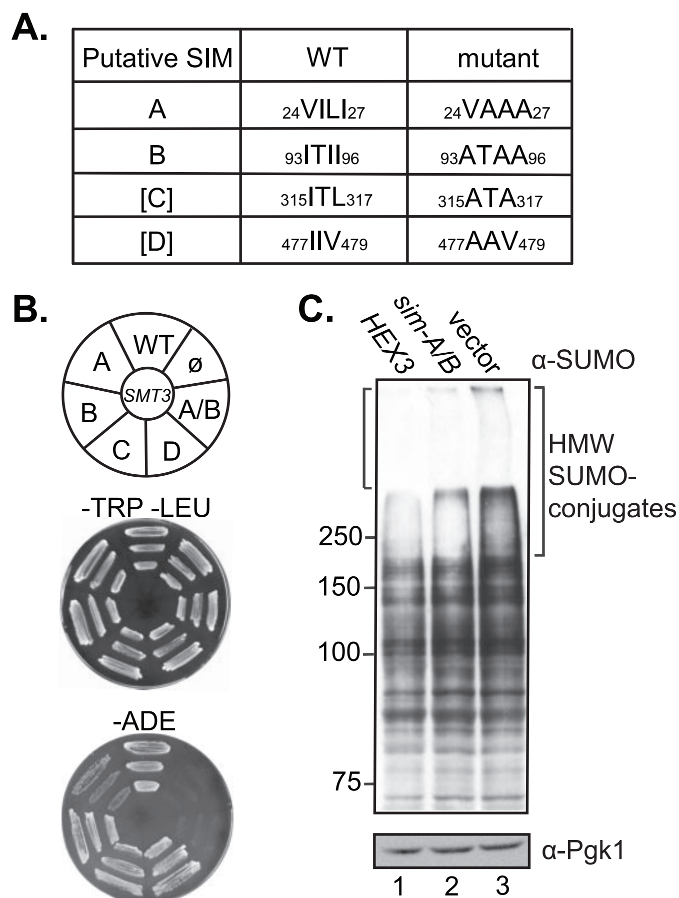

FIGURE 3. **The interaction of Hex3 with SUMO involves two SIMs.** *A*, potential Hex3 SIMs and their mutated sequences tested by yeast two-hybrid assays. *Numbers* denote positions in the Hex3 sequence. *B*, *top*, sectors of plate indicate the different prey constructs (WT, *sim-A*, *sim-B*, *sim-C*, *sim-D*, *sim-A/B*, and empty vector) tested for interactions with the Smt3/SUMO bait. The presence of both *SMT3* (pOBD2/TRP1) and *HEX3* (pOAD/LEU2) constructs was confirmed by growth on medium lacking tryptophan and leucine (*top plate*). Interaction between Hex3 and SUMO results in growth of cell patches (in triplicate) on medium lacking adenine (*bottom plate*). *C*, SUMO conjugates in *hex3*Δ cells transformed with a low-copy plasmid carrying WT *HEX3*, the *sim*-A/B *HEX3*, or no insert. Immunoblot analysis was done with anti-SUMO antisera; blots were re-probed with anti-Pgk1 to verify equal protein loading. *HMW*, high molecular weight.

matches (SIM-C and SIM-D) (Fig. 3*A*, *WT column*). To test the role of these putative SIMs in Hex3-SUMO interaction, we mutated each of them by substituting multiple hydrophobic residues with alanines (Fig. 3*A*, *mutant column*). Interactions between Hex3 mutants and SUMO were then tested by the yeast two-hybrid assay. Disruption of SIM-B, -C, or -D individually did not affect SUMO binding, but the SIM-A mutation partially impaired Hex3-SUMO interaction as measured by cell growth on SD−adenine plates. Simultaneous mutation of SIM-A and SIM-B greatly reduced Hex3-SUMO association by this assay (Fig. 3*B*). The simA/B in Hex3 did not cause reduced expression of the protein relative to WT Hex3 (supplemental Fig. S2*A*). We note that purified recombinant Hex3-simA/B could still bind SUMO *in vitro* (not shown), so additional SUMO binding sites must exist in the protein. These data indicate that Hex3 uses partially redundant SIMs, SIM-A and SIM-B, as well as additional elements to interact with SUMO and/or sumoylated proteins.

To test if the *hex3-sim*-A/B double mutant exhibited any functional defects *in vivo*, plasmids expressing WT or *sim*-A/B mutant Hex3 were transformed into *hex3*Δ cells. The *hex3-sim*-A/B mutant complemented *hex3*Δ for hydroxyurea and UV resistance (data not shown). However, compared with wild-type cells, *hex3-sim*-A/B cells displayed increased levels of high molecular weight SUMO conjugates (Fig. 3*C*). The modest inhibition of Hex3 function by the sim-A/B mutations is consistent with the failure of these mutations to fully block SUMO binding.

The RING Domains of Hex3 and Slx8 Are Required for UV and Hydroxyurea Resistance—As previously noted, both Hex3 and Slx8 contain potential zinc-coordinating RING domains, which are often associated with ubiquitin and SUMO ligases (2, 8, 12, 27). It has been reported that the RING of Slx8 is essential in *sgs1*Δ cells, suggesting that this RING contributes to Slx8 function in this context (9). We tested the requirement for the RING domains of both Slx8 and Hex3 when cells were subjected to DNA damage stress. Two predicted zinc-coordinating cysteines were changed to serines in each protein RING domain (Fig. 4*A*). Plasmid-borne alleles encoding the RING mutants or wild-type proteins were introduced into *hex3*Δ or *slx8*Δ cells. The transformants were then tested for growth after UV irradiation or on hydroxyurea (*HU*)-containing medium (Fig. 4, *B* and *C*).

As expected, WT Hex3 and Slx8 expressed from either low (CEN) or high-copy (2μ) plasmids fully complemented the growth defects of the respective deletion strains. In contrast, the RING-mutated alleles failed to rescue the *hex3* or *slx8* null mutants (Fig. 4, *B* and *C*). The *hex3-SS* and *slx8-SS* alleles were tested on high copy (2μ) plasmids to allow their detection as epitope-tagged proteins (supplemental Fig. S2), but even when expressed at higher levels than the tagged WT proteins they failed to complement the corresponding *hex3*Δ and *slx8*Δ mutations (as was true for the CEN plasmid-borne mutant alleles; data not shown). Because we had identified *HEX3* as a suppressor of *ulp1* mutants, we also tested whether overexpression of a *HEX3* RING domain truncation in combination with *SMT3gg* rescued growth of the *ulp1*Δ strain. As shown in Fig. 4*D*, it did not. These data raise the possibility that high-copy *HEX3* suppression of both DNA damage sensitivity and the *ulp1* cell division defect may occur by related mechanisms.

Slx8 Exhibits RING-dependent Auto-ubiquitination Activity—Given the presence of RING domains in both Hex3 and Slx8, we determined whether they might have SUMO-ligase or ubiquitin-ligase activity. Because of their genetic links to the SUMO pathway, we first examined potential SUMO ligase function. Both purified recombinant proteins enhanced polySUMO chain formation by low concentrations of SUMO E1 (Aos1/Uba2) and E2 (Ubc9) (supplemental Fig. S3*A*). Although these data suggested that Hex3 and/or Slx8 might have significant polySUMO chain-stimulating activity, in neither case did this activity require an intact RING domain (supplemental Fig. S3*A*), so its physiological relevance remains unclear. We also tested *in vitro* sumoylation of the DNA repair protein Rad52, which is sumoylated *in vivo* (see below). Even at very high E3:substrate ratios (~10:1), only minimal modification was seen, and a control that mixed a known SUMO E3, Siz1, with Slx8 showed more activity than did Hex3-Slx8 (supplemental

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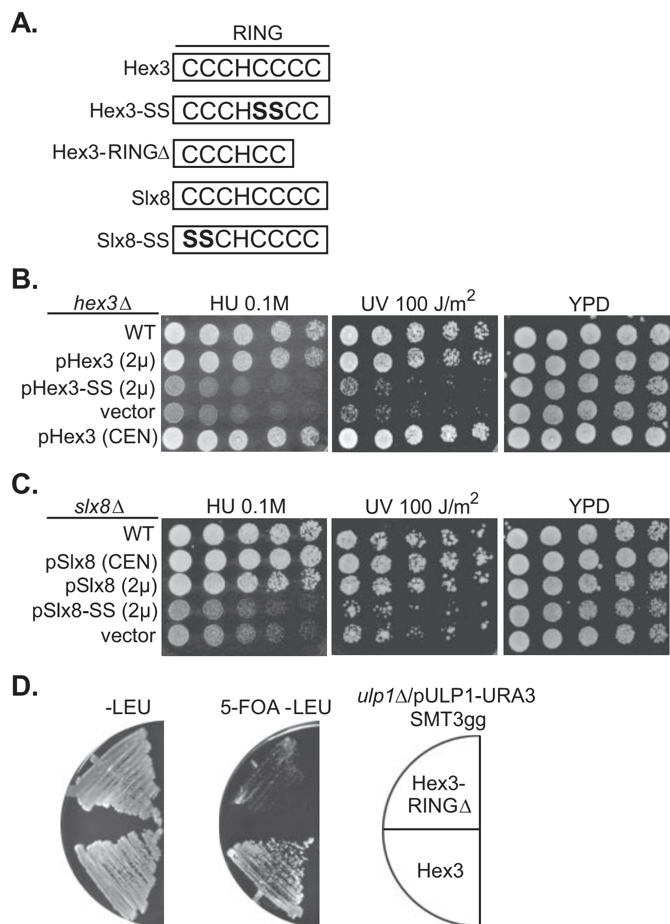

FIGURE 4. **The RING domains of Hex3 and Slx8 are required for cellular DNA damage resistance.** *A*, schematic of WT and mutant RING domains of Hex3 and Slx8 tested in this study. Conserved cysteine (*C*) and histidine (*H*) residues are shown in *light face*; serine (*S*) mutations are shown in *bold*. *B*, mutant *hex3*Δ cells transformed with the indicated plasmids were grown under several different conditions and compared with WT cells. *YPD*, yeast extract/peptone/dextrose. *C*, mutant *slx8*Δ cells were transformed with the indicated plasmids, and growth phenotypes of serially diluted transformants were compared with WT cells after UV damage or on plates containing hydroxyurea (*HU*). *D*, WT Hex3 and Hex3-RINGΔ were expressed from 2μ *LEU2* plasmids in a *ulp1*Δ/pULP1-URA3 strain also expressing *SMT3gg*. The ability of overexpressed Hex3 and Hex3-RINGΔ to suppress the lethal *ULP1* deletion was tested on 5-fluoro-orotic acid (*5-FOA*).

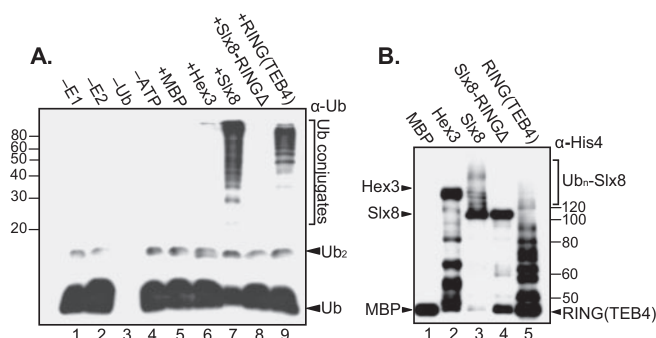

FIGURE 5. **Slx8, but not Hex3, exhibits self-ubiquitination activity *in vitro*.** *A*, purified recombinant Hex3, Slx8, Slx8-RINGΔ, MBP, and RING(TEB4) were used in an *in vitro* auto-ubiquitination (*Ub*) assay. *Lane 5–9*, the recombinant proteins listed were mixed in a reaction buffer containing E1 (Uba1), E2 (Ubc4), ubiquitin, and ATP. Control reactions are depicted in *lanes 1–4*. Samples from individual reactions were resolved by 16.5% Tricine SDS-PAGE and immunoblotted with anti-ubiquitin antibodies (α-*Ub*). The purified RING domain of TEB4 was included as a positive control (*lane 9*). Mono-, di-, and higher order ubiquitin conjugates are indicated. *B*, samples from *A* were resolved on an 8% SDS-PAGE gel and immunoblotted with an anti-His4 antibody to detect the purified His_6-tagged proteins shown.

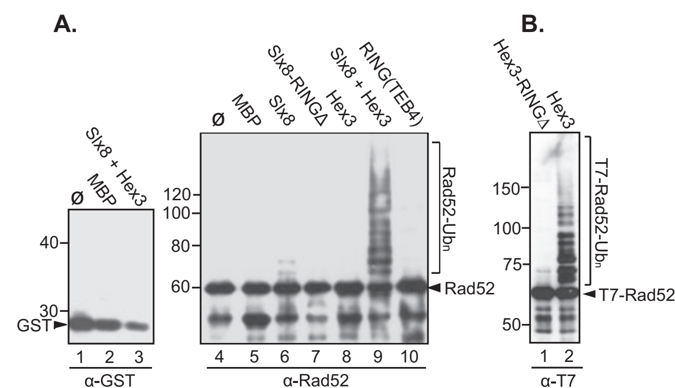

FIGURE 6. **Yeast Rad52 protein is a substrate for Hex3·Slx8-mediated ubiquitination.** *A*, recombinant yeast Rad52 (0.2 μM) was incubated at 30 °C for 2 h with E1 (*Uba1*), E2 (*Ubc4*), and 0.4 μM of the indicated recombinant proteins (*lane 5–10*). Control reactions lacking E3 or with MBP (*lanes 4–5*) or containing purified GST in place of Rad52 (*lane 1–3*) are also shown. *B*, equal amounts (0.4 μM) of recombinant Hex3 or Hex3-RINGΔ were supplied to separate ubiquitination reactions containing Uba1, Ubc4, Slx8, and 0.2 μM T7-tagged recombinant Rad52. Reaction products were examined by anti-T7 immunoblotting. *GST*, glutathione *S*-transferase.

Fig. S3*B*). The increase in levels of high molecular weight SUMO conjugates in *hex3*Δ and *slx8*Δ yeast (Fig. 1*D*), instead of a decrease, also appears inconsistent with a general role for the two proteins as canonical SUMO ligases.

We, therefore, assayed Hex3 and Slx8 for potential ubiquitin ligase activity. Both proteins were analyzed by an *in vitro* auto-ubiquitination assay with E1 (Uba1) and E2 (Ubc4). The reaction products were analyzed by anti-ubiquitin immunoblotting (Fig. 5*A*). Notably, Slx8 catalyzed formation of large ubiquitinated species (Fig. 5*A*, *lane 7*), but Hex3 did not (*lane 6*). The Slx8-RINGΔ mutant also failed to stimulate ubiquitin conjugate formation (*lane 8*). At least a fraction of the ubiquitinated species represented Slx8-ubiquitin conjugates (Fig. 5*B*, *lane 3*). These data were similar to the positive control, the RING of a known ubiquitin E3, TEB4 (28). Neither Hex3 nor the Slx8-RINGΔ proteins became ubiquitin-modified. Together, these data indicate that Slx8 has a RING-dependent self-ubiquitinating activity similar to that of other RING E3 ubiquitin ligases.

Hex3 Facilitates Slx8-directed Ubiquitination of Yeast Rad52 Protein—A growing body of genetic data has implicated Hex3 and Slx8 in the control of DNA damage (8, 10, 29). The results presented above suggest that Slx8, potentially with Hex3, may help control DNA damage through a ubiquitin ligase activity. Conceivably, substrates of Slx8 could be regulators or effectors of the DNA-damage response. Therefore, we tested several candidate substrates of Hex3·Slx8 for *in vitro* ubiquitination. Of several purified proteins tested, the Rad52 protein, which functions in DNA repair and homologous recombination, was found to be weakly ubiquitinated by Slx8 (Fig. 6, *lane 6*) but not by Hex3 alone (*lane 8*) or by the truncated Slx8-RINGΔ protein (*lane 7*). Activity was greatly stimulated by the addition of Hex3 (*lane 9*). The identity of the slower migrating Rad52-reactive bands in Fig. 6*A* as multiubiquitinated species was confirmed by their reduced mobility when hemagglutinin-tagged ubiquitin replaced ubiquitin in the reaction (data not shown). The RING domain of TEB4 did not ubiquitinate Rad52 (Fig. 6*A*, *lane*

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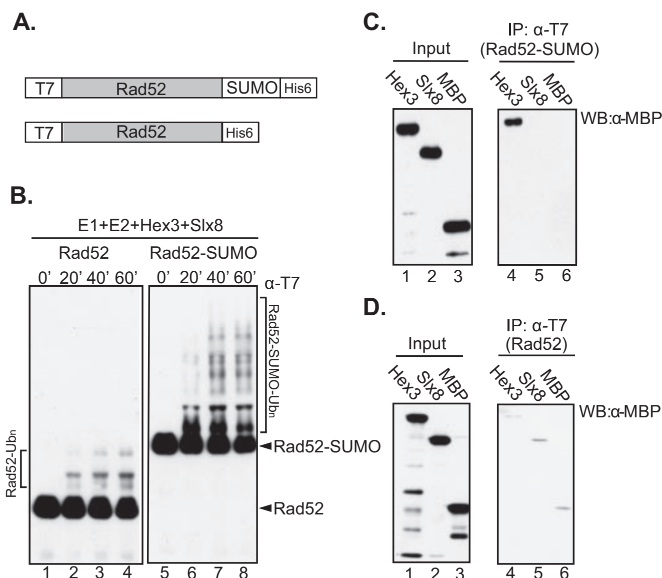

FIGURE 7. **Hex3·Slx8 is a SUMO-directed ubiquitin ligase for Rad52.** *A*, schematic of T7-tagged Rad52 and Rad52-SUMO fusion proteins. *B*, Rad52-SUMO fusion is a better substrate than Rad52 for *in vitro* Hex3·Slx8-mediated ubiquitination. Equal amounts of recombinant Rad52 and Rad52-SUMO (0.4 μM) were used in the ubiquitination (Ub_n) reactions together with Uba1, Ubc4, and 0.2 μM each of Hex3 and Slx8. Reactions were conducted at 30 °C for the indicated times, and reaction products were examined by anti-T7 immunoblotting. *C*, co-precipitation of Hex3 with Rad52-SUMO. Rad52-SUMO was incubated with Hex3, Slx8, and MBP before immunoprecipitation with anti-T7 antibodies. Precipitated proteins were resolved by 8% SDS-PAGE followed by anti-MBP immunoblotting. *Lanes 1–3* denote 3.33% input of Hex3, Slx8, and MBP. *D*, co-immunoprecipitation (*IP*) of Hex3 and Slx8 with Rad52. The experiment is identical to that in *panel C* except that Rad52 was used instead of Rad52-SUMO.

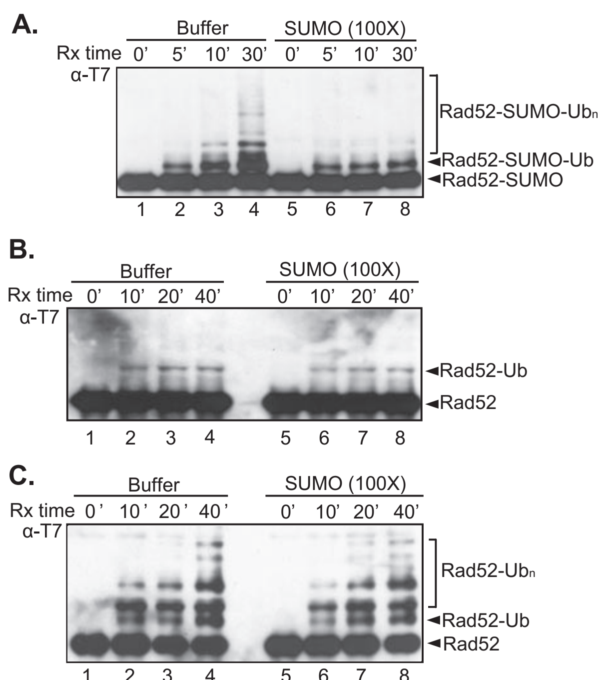

FIGURE 8. **Ubiquitination of Rad52-SUMO is specifically inhibited by excess free SUMO.** *A*, 0.2 μM each of Hex3 and Slx8 were incubated with purified SUMO present in 100-fold molar excess relative to substrate or with buffer alone for 30 min before being introduced into the Rad52-SUMO (0.4 μM) ubiquitination (Ub_n) assay. Immunoblotting was done as in Fig. 7*B*. *B*, similar free SUMO inhibition assay as in *A* except that Rad52 (0.4 μM) was used as a substrate. *C*, ubiquitination of Rad52 was carried out as *B* but with elevated amounts of Hex3 and Slx8 (Hex3·Slx8/substrate = 0.4 μM/0.2 μM). *Rx*, reaction.

10) nor was purified Rad51, another homologous recombination protein, modified by Hex3·Slx8.[7]

Given that the RING of Hex3 is required for its *in vivo* function (Fig. 4, *B* and *D*), we also tested its importance in Rad52 ubiquitination *in vitro*. Whereas wild-type Hex3 strongly activated Slx8-dependent Rad52 ubiquitination, the Hex3-RINGΔ truncation failed to enhance the reaction (Fig. 6*B*). These data are consistent with a model in which the RING domains of both Slx8 and Hex3 contribute to the ubiquitin ligase activity, with Slx8 likely functioning as the core ligase that is activated by Hex3. This arrangement is reminiscent of the human BRCA1-BARD1 RING heterodimer, in which the BRCA1 RING is the crucial E3 effector, but both polypeptides are needed for maximal activity (30, 31).

Role of SUMO in Hex3·Slx8-mediated Rad52 Ubiquitination—Hex3 both associates with SUMO (Fig. 2*C*) and enhances the Rad52-specific ubiquitin ligase activity of Slx8 (Fig. 6*A*). Notably, Rad52 becomes sumoylated after DNA damage (32, 33). We, therefore, hypothesized that Hex3·Slx8-mediated ubiquitination of Rad52 might be stimulated by Rad52-SUMO ligation. Direct translational fusion of SUMO to substrates often mimics the effects of post-translational SUMO addition to the same proteins *in vitro* and *in vivo* (34–36). We adopted this strategy and generated a Rad52-SUMO fusion that could be purified from *E. coli* and used in our *in vitro* ubiquitination system (Fig. 7*A*). When equimolar amounts of recombinant Rad52 and Rad52-SUMO were tested as Hex3·Slx8 ubiquitination substrates under suboptimal conditions, Rad52-SUMO was efficiently ubiquitinated, whereas Rad52 alone was only modified to a limited degree (Fig. 7*B*).

Rad52-SUMO might be a better ubiquitination substrate because Hex3·Slx8 can bind the SUMO moiety and enhance E3-substrate interaction. We tested this idea by comparing the ability of anti-T7 antibodies to co-precipitate T7-tagged Rad52 or Rad52-SUMO and either Hex3 or Slx8. With T7-Rad52-SUMO, only MBP-Hex3 was detectably co-precipitated, whereas MBP-Slx8 and MBP were not (Fig. 7*C*). When T7-Rad52 was precipitated, neither MBP-Hex3 nor MBP-Slx8 was detected above background (Fig. 7*D*). These data suggest that Rad52-SUMO preferentially binds to Hex3 (in Hex3·Slx8), which in turn may account for the enhanced Slx8-mediated ubiquitination of this protein.

If enhanced ubiquitination of Rad52-SUMO compared with Rad52 was due to direct E3-SUMO binding, then excess free SUMO should selectively inhibit Rad52-SUMO ubiquitin ligation. Using a 100-fold excess of free SUMO relative to substrate, ubiquitination of Rad52-SUMO was indeed impaired (Fig. 8*A*). In contrast, the same free SUMO/substrate ratio had little, if any, effect on the ubiquitination of Rad52 lacking the SUMO moiety (Fig. 8*B*). Although increasing the concentration of Hex3·Slx8 enhanced Rad52 ubiquitination, this modification was still largely unaffected by the presence of free SUMO (Fig. 8*C*). We note that the Hex3-simA/B mutant, which can still bind SUMO *in vitro*, also still could stimulate Rad52-SUMO

[7] P. Chi and P. Sung, unpublished data.

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ubiquitination (data not shown). In summary, our findings suggest that the Hex3·Slx8 complex is a substrate-specific ubiquitin ligase that is stimulated by prior attachment of SUMO to the substrate.

DISCUSSION

In this study, we have demonstrated that the Hex3·Slx8 complex functions as a ubiquitin-protein ligase (E3). Full activity requires the RING domains of both Hex3 and Slx8, but it appears that Slx8 possesses the core ligase activity. Hex3 enhances E3 activity toward its substrates. Surprisingly, one way by which Hex3 appears to stimulate substrate ubiquitination is by binding conjugated SUMO in a target protein. This is the first example of such a mechanism of substrate-specific cross-talk between the SUMO and ubiquitin systems but is likely to be utilized more broadly. Related findings on the Hex3-Slx8 ubiquitin ligase are published in the accompanying article by Uzunova *et al.* (50).

There are two examples in higher organisms of heterodimeric RING proteins catalyzing substrate ubiquitination, BRCA1-BARD1 and RING1a/b-BMI1 (30, 31, 37, 38). Like Hex3-Slx8, the BRCA1-BARD1 complex also functions in the control of DNA damage (39, 40). Mutation of the RING domain in either Hex3 or Slx8 results in a null phenotype in every assay done to date (Ref. 9; this study). Because Slx8 by itself has a robust auto-ubiquitinating activity as well as a weak activity in the trans-ubiquitination of Rad52, Slx8 presumably interacts directly with the E2 enzyme Ubc4 to promote ubiquitination, as in a canonical ubiquitin RING E3 (2, 41). The exact function of the Hex3 RING is less clear. These findings are also reminiscent of the BRCA1-BARD1 RING heterodimer, where the BRCA1 RING but not BARD1 binds UBCH5c, which is in the same E2 subfamily as yeast Ubc4 (14). Similar findings have been reported for the RING1a/b-BMI1 RING protein heterodimer, where the BMI1 protein has no detectable intrinsic E3 activity, although its RING is essential for activity of the complex (42, 43). For Hex3 as well as BARD1 and BMI1, the RING might stabilize an active conformation of the partner RING.

One of the more remarkable observations of the current study is that modest overproduction of the Hex3 protein allows for a complete bypass of the normally essential role played by the Ulp1 SUMO isopeptidase in cell cycle progression (Fig. 1*A* and supplemental Fig. S1*A*). Because there is no detectable alteration to bulk SUMO-conjugate patterns under these conditions (data not shown), it is unlikely that Hex3 or a Hex3-regulated factor functions as a SUMO protease that can substitute for Ulp1. In Fig. 9, we present a general model for the function of Hex3-Slx8 and the suppression of *ulp1*Δ by increased levels of the Hex3-Slx8 heterodimer. Specifically, we suggest that a protein(s) (X) is sumoylated and that in this modified form it more strongly inhibits progression through the G_2/M phase of the cell cycle. Ulp1 can counteract this inhibitory mechanism by desumoylating the X protein(s). Alternatively, upon increasing levels of Hex3, which we assume is present in limiting amounts relative to Slx8, enhanced ubiquitination of the inhibitory sumoylated factor leads to its inactivation, presumably by proteasomal degradation. Mutant *hex3* and *ulp1* strains share several characteristics, including a "nibbled" colony morphology, elevated frequencies of recombination, and sensitivity to certain DNA damaging agents (8, 29, 44, 45). The model in Fig. 9 predicts that Ulp1 and Hex3·Slx8 have overlapping functions in the cell cycle and in controlling the accumulation of DNA damage. This view is consistent with the strong synthetic defects observed in the *ulp1ts hex3*Δ double mutant (supplemental Fig. S1*B*).

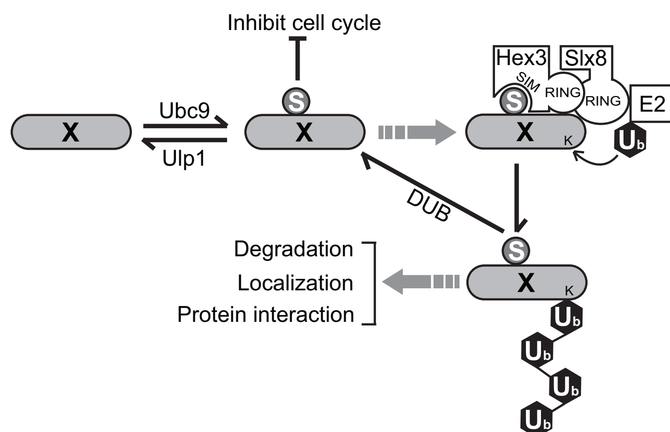

FIGURE 9. **Proposed model for Hex3·Slx8 function.** For details, see "Discussion."

Our results support the idea that SUMO attachment to protein substrates provides the nexus where regulation by Ulp1 and Hex3-Slx8 directly intersect. This is self-evident for desumoylation by Ulp1, but the recruitment of a ubiquitin ligase to a substrate by prior substrate sumoylation has not been reported previously. The *in vitro* data suggest that direct Hex3-SUMO binding is responsible for facilitating association of the Hex3·Slx8 ligase with the sumoylated target protein. Two partially redundant SIMs in Hex3, SIM-A and SIM-B, contribute to SUMO binding. Despite the importance of SIM-A and SIM-B for SUMO association by the two-hybrid assay, the only phenotypic defect we detected with the *hex3-sim-*A/B mutant was an enhanced accumulation of high molecular mass SUMO conjugates. Residual SUMO or polySUMO binding presumably still occurs with the mutant Hex3 protein *in vivo* as it does *in vitro*. It is also clear that Hex3·Slx8 is active *in vitro* toward nonsumoylated substrates, at least at high concentrations (Fig. 6), so it is possible that low SUMO-independent activity is sufficient under many circumstances *in vivo*.

We identified Rad52 as an *in vitro* substrate of the Hex3·Slx8 ubiquitin ligase. Interestingly, a recent study reported genetic interactions between mutations in *HEX3/SLX8* and *RAD52* that are consistent with the Hex3·Slx8 complex negatively regulating Rad52-dependent recombination when such activity would induce DNA damage (29). Our model (Fig. 9) would predict enhanced Hex3·Slx8-mediated ubiquitination of sumoylated Rad52. Rad52 is strongly sumoylated *in vivo* after certain types of DNA damage and can be degraded by the ubiquitin-proteasome pathway (32).[8] However, neither Rad52 nor a Rad52-SUMO fusion was degraded more slowly in *hex3*Δ or *slx8*Δ cells than in WT cells (data not shown). It remains possible that Hex3·Slx8-mediated Rad52 ubiquitination does not

[8] Y. Xie, O. Kerscher, and M. Hochstrasser, unpublished results.

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lead to its degradation or that such modification only affects a specific fraction of Rad52, which we failed to observe. By genetic criteria, Hex3 and Slx8 appear to have only a partial functional overlap with the Rad52-dependent repair pathway (29, 46, 47). We suspect that the Hex3·Slx8 E3 can ubiquitinate other substrates that participate in DNA damage control and predict that they are also conditionally sumoylated.

Apparent homologs of Hex3 and Slx8 are found in *Schizosaccharomyces pombe* and higher organisms, including humans (8, 48). The human RNF4 protein has ubiquitin ligase activity and can also bind SUMO noncovalently (49). Whether such binding stimulates its ubiquitin ligase activity toward specific sumoylated substrates is not yet known. However, it seems likely that our proposed model for yeast Hex3·Slx8 regulation and activity will be more broadly applicable. The BARD1 protein also has several good matches to the SIM consensus sequence; the possibility that the BARD1-BRCA1 ubiquitin ligase is also regulated by substrate sumoylation is intriguing given the other similarities with yeast Hex3-Slx8 noted above. A thorough understanding of the mechanistic and physiological functions of Hex3·Slx8 and its homologs will require further biochemical and biophysical analysis of their SUMO-dependent and -independent ubiquitin ligase activities as well as identification and characterization of their natural *in vivo* substrates.

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